

(19)



Europäisches Patentamt
Europäischer Patentamt
European Patent Office
Office Européen des brevets



(11)

EP 0 844 252 A2

(12)

EUROPEAN PATENT APPLICATION

(43) Date of publication:
27.05.1998 Bulletin 1998/22

(51) Int Cl. 6: C07K 7/64, C07K 17/00

(21) Application number: 97870182.9

(22) Date of filing: 14.11.1997

(84) Designated Contracting States:
AT BE CH DE DK ES FI FR GB GR IE IT LI LU MC
NL PT SE
Designated Extension States:
AL LT LV MK RO SI

(30) Priority: 15.11.1996 US 30980 P

(71) Applicant: Remacle, José
5020 Malonne (BE)

(72) Inventors:

- Remacle, José
5020 Malonne (BE)
- Delforge, Dominique
5000 Namur (BE)

(74) Representative: Van Malderen, Joelle et al
Office Van Malderen,
Place Reine Fabiola 6/1
1083 Bruxelles (BE)

(54) **Cyclic peptides bearing a tail designed for subsequent chemical coupling and process for preparing same**

(57) The present invention relates to a cyclic peptide comprising a dicarboxylic amino acid, said cyclic peptide bearing at least one tail for subsequent coupling on a solid support, on a high molecular weight compound, on a marker, or on one or more other similar or different cyclic peptides.

The present invention is also related to the preparation process of said cyclic peptide and to a biomaterial comprising said cyclic peptide.

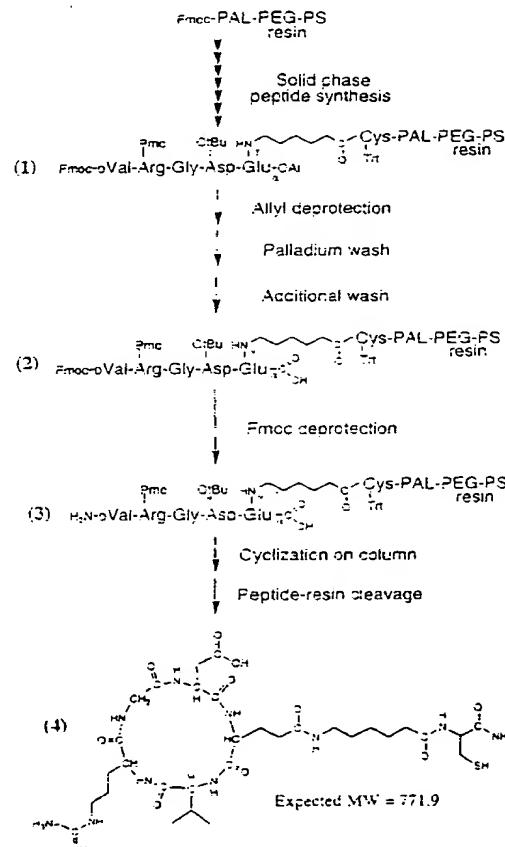


FIG. 1

Description**Field of th** invention

The present invention is in the field of the biotechnology and relates to cyclic peptides designed for subsequent chemical grafting or coupling on a solid support, on a marker, on a high molecular weight compound and/or another cyclic peptide, and to their preparation process.

Background of the invention and state of the art

Peptides play a primordial role *in vivo* because of their ubiquitous implication in a variety of interactions with other biological molecules and proteins such as cell receptors, antibodies or enzymes. Although they are naturally flexible and can adopt a large number of different conformations, their biological activity is usually the result of one specific three-dimensional conformation. In laboratory experiments the conformation of peptides is also studied to reproduce specific determinants (epitopes) of high molecular weight proteins. The length of the peptides used for these studies is in general limited (up to fifteen amino acids) to overcome the problems that arise with increasing length of the peptide.

However, these peptides show a higher flexibility compared to the native protein. For this reason, their biological activity is usually lower than in the native conformation unless they are in some way rigidified. One way to achieve this is to synthesise different head-to-tail cyclic peptides with restricted conformational flexibility and to determine which of these analogs retains biological activity (Kessler H., 1982, Angew. Chem. Int. Ed. Engl., 21, 512-523; Kessler H *et al.*, 1989, in Computer-aided drug design, methods and applications, Ed T.J. Perun and C.L. Probst, pp. 461-484, Marcel Dekker, New-York; Hruby V.J. *et al.*, 1990, Biochem. J., 268, 249-262; Toniolo C., 1990, Int. J. Pept. Protein Res., 35, 287-300; Gilon C *et al.*, 1991, Biopolymers, 31, 745-750).

Cyclic peptides can be formed in solution, in diluted conditions, by activation of a side-chain protected linear peptide using carbodiimide or diphenylphosphorylazide, followed by subsequent deprotection of the side-chains (Kessler H *et al.*, 1989, in Computer-aided drug design, methods and applications, Ed. T.J. Perun and C.L. Probst, pp. 461-484, Marcel Dekker, New-York; Toniolo C., 1990, Int. J. Pept. Protein Res., 35, 287-300; Gurrath M. *et al.*, 1992, Eur. J. Biochem., 210, 911-921; Izumiya N. *et al.*, 1981, Biopolymers, 20, 1785-1791; Brady S.F. *et al.*, 1983, in Peptides. Structure and Function, Proceedings of the Eighth American Peptide Symposium, Ed. V.J. Hruby and D.H. Rick, pp. 127-130, Pierce Chemical Company, Rockford, Illinois; He J.X. *et al.*, 1994, Lett. Peptide Sci., 1, 25-30). Side-chain to side-chain cyclization has been achieved through lactam (Hruby V.J. *et al.*, 1990, Biochem. J., 268,

249-262; Hoffmann E. *et al.*, 1991, Liebigs Ann. Chem., 585-590) or disulphide bridge formation, between two cysteines incorporated in the sequence (Hruby V.J. *et al.*, 1990, Biochem. J., 268, 249-262; Cavelier F. *et al.*,

5 1989, Bull. Soc. Chim. France, 788-798). Cyclization of unprotected peptides has also been obtained by oxime formation in aqueous solution (Pallin T.G. and Tam J.P., 1995, J. Chem. Soc., Chem. Commun., 2021-2022). Although these methods have been largely used they suffer from the fact that they are time-consuming and that side reactions like cyclodimerization, oligomerization or racemization of the C-terminal residue are difficult to avoid.

To overcome this problem, a fully automated synthesis procedure has been proposed, with the cyclization step occurring directly on the column (Kates S.A. *et al.*, 1993, Anal. Biochem., 212, 303-310). This method allows the synthesis of cyclic peptides containing an acid (Asp, Glu) or an amide residue (Asn, Gln) (Kates S.A. *et al.*, 1993, Anal. Biochem., 212, 303-310). In this method, an α -allyl protected acid residue like Fmoc-Asp-OAI or Fmoc-Glu-OAI is incorporated as the first residue during the synthesis by fixation of its free carboxylic side-chain on the synthesis support. The peptidic chain is then elongated following the standard Fmoc synthesis procedure before specific cleavage of the α -allyl protecting group, at the carboxyterminal end of the peptide, with palladium (0). Then the nucleophilic attack of the amino-terminal group of the peptide on the deprotected carboxylic end, allows the head-to-tail cyclization on the column (Kates S.A. *et al.*, 1993, Anal. Biochem., 212, 303-310).

Other strategies incorporating (bromoacetyl) diamino-propionic acid in the sequence (Ivanov *et al.*, 1995, Bioconjugate Chem., 6, 269-277) or bromoacetyl-beta-alanyl-lysine derivatives (US 5444150) are also described in the scientific literature. Cyclization was for example obtained by covalent linkage between diamino-propionic acid and a cysteine residue of the sequence. 40 This cyclization method was not automated and was achieved by a side-chain to side-chain link.

Aims of the invention

45 The present invention aims to provide cyclic peptides and their preparation process, which allow subsequent chemical grafting or coupling on said cyclic peptides, i.e. their attachment to a solid support, to a high molecular weight compound, to a marker and/or to other 50 cyclic peptides, in order to provide new or improved biotechnological applications, particularly in the field of affinity chromatography, immunisation, development of diagnostic tests, vaccines and pharmaceutical compositions, for Biacore experiments, for development of libraries of various conformational constrained peptides or for the development of new biomaterials which allow or improve the binding of specific antibodies, receptors, ligands, cells, tissues

Summary of the invention

The present invention is related to a cyclic peptide comprising a dicarboxylic amino acid. said cyclic peptide bearing at least one tail for subsequent coupling on a solid support, on a high molecular weight compound, on a marker and/or on one or more other (similar or different) cyclic peptides.

It is meant by a "cyclic peptide", a peptide comprising 4 or more amino acids (preferably between 4 and 15 amino acids) linked together to form a cycle, wherein at least one of said amino acids is a dicarboxylic amino acid.

However, the cyclic peptide according to the invention may also comprise amino acids which are coupled or grafted to other chemical compounds, such as saccharides (polysaccharides, oligosaccharides, monosaccharides, made of glucose, fructose, mannose, fucose, ribose, ... units).

Advantageously, the dicarboxylic amino acid included in the cyclic peptide according to the invention is a (D or L) glutamic acid or a 2-amino adipic acid (D or L).

It is meant by a "tail", a chain or an arm made of chemical compounds, which allows the coupling of the cyclic peptide according to the invention on a solid support, on a high molecular weight compound, on a marker and/or on other cyclic peptides.

Advantageously, said tail can be branched in order to obtain the coupling of the cyclic peptide to several solid supports, several high molecular weight compounds, several markers and/or several cyclic peptides.

It is meant by "a high molecular weight compound", a molecule such as a protein carrier (BSA, KLH, lysosome, ...) having a molecular weight higher than 5 KD.

It is meant by "a marker", any chemical compound, such as a fluorescent chemical compound, a radioactive compound, which allows the specific identification of the cyclic peptide according to the invention.

According to a preferred embodiment of the present invention, the tail of the cyclic peptide is made of amino acids units such as cysteine, lysine, aspartic acid or glutamic acid, possibly separated by one or more spacers.

It is meant by a "spacer between the various amino acids units of the tail", one or more organic compounds which can link two or more amino acids such as amino acids having the formula -NH₂-alkyl-COOH or -NH₂-aryl-COOH. Preferably, said spacer is a 6-amino-hexanoil.

The amino acids present in the cycle of the cyclic peptide according to the invention are natural amino acids or non-natural amino acids (other amino acids than the 20 amino acids used in the build up of natural proteins).

Preferably, said amino acids of the cycle of the cyclic peptide according to the invention are building blocks such as the ones described by James G.L. et al.

(1993, Sciences, 260, 1937-1942), William B.J. et al. (1993, J. Med. Chemistry, 31), Ward P. et al. (1990, J. Med Chemistry, 33, 1848) or templates as described by Jackson S. et al. (1994, J. Am. Chem. Soc., 116, 3220-3230) used for the synthesis of peptido-monomers.

According to a specific embodiment of the present invention, the cyclic peptide according to the invention comprises in its cycle a sequence which mimics one binding sequence of a growth factor, preferably the sequence SEQ ID NO. 1 : EQIMRIKPHQ.

The present invention is also related to the solid support coupled to the cyclic peptide according to the invention. Preferably, said solid support is selected from the group consisting of a chromatographic gel (of a chromatography column), beads, the surface of any container such as a multiwell plate, preferably an ELISA polystyrene multiwell plate, or any other solid support which can be used for the culture of cells.

Another aspect of the present invention is related to the high molecular weight compound such as a protein carrier (BSA, KLH, lysosome, ...) coupled to the cyclic peptide according to the invention.

Another aspect of the present invention is related to the biomaterial comprising the high molecular and/or the solid support coupled to the cyclic peptide according to the invention, which allows adhesion, preferably a selective adhesion, of specific cells and/or tissues. Said biomaterial comprising said cells and/or tissues grafted or fixed upon the cyclic peptide according to the invention, and which can be incorporated into a biosensor, a bioreactor, an artificial organ or an artificial tissue.

According to the composition of the various amino acids of the cycle of said cyclic peptide, said biomaterial according to the invention can be used for the grafting or binding of specific antibodies, receptors, ligands, cells or tissues, and may be used for the production or the identification of known or new drugs, vaccines, hormones, interferons, cytokines, etc.

The biomaterial according to the invention may also be used for the screening of specific cells, in particular for the diagnostic of tumour cells, which may adhere differently than the normal cells upon the various types of the biomaterial according to the invention.

The cells (or tissues) above-described can be of vegetal, animal and fungi origin, or can be unicellular micro-organisms.

The biomaterial according to the invention can also be used to allow preferential cells and/or tissues adhesion on specific parts of the biomaterial in order to guide the cells and tissue growth along preferential directions, particularly for specific applications such as nerve reconstruction.

It is clear that the biomaterial according to the invention may also comprise specific mediums or compounds, such as extra-cellular matrix proteins or a portion thereof, which allow the proliferation of specific cells or tissues upon said biomaterial.

Examples of extra-cellular matrix proteins are collagen, laminin, fibronectin, fibrin, chondronectin or a mixture thereof, as described in the Patent Application WO96/09372, incorporated herein by reference.

The biomaterial according to the invention may also comprise specific mediums well known by the person skilled in the art and which will allow the survival and/or the proliferation of specific cells upon the biomaterial according to the invention (for instance, DMEM medium obtained from Bio Whittaker, which allows the proliferation of Schwann cells, the William's E medium obtained from Sigma, which allows the proliferation of hepatocytes, etc.).

The solid support of the biomaterial according to the invention can also be a microporous polymeric membrane covered upon one or both of its sides by one or more different cells strains in a specific nutritive medium, such as the "system" described in the International Patent Application WO96/09372, incorporated herein by reference.

A last aspect of the present invention is related to the process for the preparation of the cyclic peptide according to the invention, which is advantageously obtained by chemical synthesis using an automatic peptide synthesiser, preferably by a chemical synthesis directly on a solid support or a high molecular weight compound.

Preferably, in the method according to the invention, the washing conditions comprise the use of dichloromethane as a solvent in the presence of an aminated base in an additional washing step, after the steps of allyl cleavage and palladium wash.

Alternatively, the cyclisation of the peptide could be achieved by replacing the Fmoc-Glu-Oal compound by Fmoc-Glu-ODmab and performing the cleavage step of Dmab protecting groups by lowing 2% hydrazine monohydrate in DMF through the resin (60 minutes) and subsequent washing of the resin with 8% DIEA in DMF. The resin cyclisation was achieved by treatment of the resin either with diisopropylcarbodiimide (DIPCDI)/HOAE/DIEA in a fourfold excess for 2 x 18 hours, or with PyAOP/DIEA (fourfold excess) for 2 x 5 hours.

The present invention will be described in several non-limiting examples in reference to the following figures.

Short description of the drawings

The Figure 1 represents schematically the method used for automated synthesis of the tailed-cyclic peptide *cyclo[D-Val-Arg-Gly-Asp-Glu(-εAhx-Cys-NH₂)-]* (allyl deprotection = Palladium(0) in acetic acid 5% / NMM 2.5% / chloroform (syringe 3); Palladium wash = DIEA 0.5% / sodium diethyldithio-carbamate 0.5% / DMF (auxiliary bottle); Additional washing = DIEA 8% in CH₂Cl₂

5

The Figure 2

10

(wash 2 bottle): Fmoc cleavage = Pipеридине 20% in DMF (deblock bottle); Cyclization on column = TBTU, BOP or HATU 0.3 M in DIEA 8% / DMF (syringe 2); Peptide-resin cleavage = TFA : thioanisole : ethanedithiol : anisole (90:5:3:2))

represents a reversed-phase HPLC profile of the crude product obtained after synthesis of the tailed cyclic peptide *cyclo[D-Val-Arg-Gly-Asp-Glu(-εAhx-Cys-NH₂)-]* according to Fig. 1 with an additional washing step after the Palladium wash (A. Manual washing step and cyclization with HATU (3 x 60 min); B. Automatic additional washing step and cyclization with TB-TU reagent (2 x 3 h); C. Automatic additional washing step and cyclization with HATU reagent (2 x 60 min). Molecular weight of eluting compounds (ESI-MS): (4) = 771; (10) = 1543.6).

represents the grafting of ¹²⁵I-labelled tailed cyclic peptide on aminated PET,

with (gray histograms) or without (white histograms) SMCC as coupling reagent (CTL = grafting on native (unmodified) PET films: test = grafting on aminated PET films).

20

The Figure 3

25

represents a (A) schematic representation of the method used for grafting tailed cyclic RGD peptides on BSA, its adsorption on polystyrene plates and its use for adhesion assays, and (B) a schematic representation of the method used for grafting tailed cyclic RGD peptides on BSA after it has been adsorbed on the polystyrene plate.

30

The Figure 4

35

shows the results of the adhesion of endothelial cells on reconditioned polystyrene support as described in Fig. 4A (the albumin was first grafted with the tailed cyclic RGD peptide before being adsorbed on the plate. The assay was performed on fibronectin coated plates (FN), on BSA coated plates (BSA), on BSA coated plates grafted with cysteine (BSA-CYS), with a cyclic RGD peptide (BSA-T26)).

40

The Figure 5

45

shows the results of the adhesion of endothelial cells on reconditioned polystyrene supports as described in Fig. 4B (the assay was performed on fibronectin coated plates (FN), on polystyrene (PS), on BSA coated plates (BSA), on BSA coated plates grafted with cysteine (BSA-CYS), with a linear RGD peptide (BSA-P21), with a cyclic

50

The Figure 6

55

RGD peptide (BSA-T26) or with a cyclic DRVG peptide (BSA-T29). Adhesion of the cells was estimated after 15 min incubation using a colorimetric assay).

The Figure 7

shows the results of same experiment as in Fig. 6 wherein the peptides were directly adsorbed on the BSA-coated plates without coupling reagent.

The Figure 8

represents the structure of a dimeric tailed cyclic peptide which mimic VEGF and could act as agonist of this growth factor for the binding to KDR receptor.

Detailed description of the invention

The tailed cyclic peptide according to the invention is a peptidic sequence which contains a dicarboxylic amino acid, like glutamic acid, which is linked in the peptidic cycle part of the peptide by its α-carboxylic and amino group, and bears the anchoring tail on its side-chain carboxylic group. One example is given in Fig 1 which summarises the procedure followed to obtain a five amino acid peptide *cyclo*(-D-Val-Arg-Gly-Asp-Glu-) with an anchoring tail 6-aminohexanoic acid and cysteine. The choice of this tail composition is based on the fact that the 6-aminohexanoic acid introduces a 6 atom long tail which is usually sufficient to allow the peptide to be far enough from the solid support and to be recognised by a binding constituent like a receptor, an antibody, etc. If necessary other amino acids or longer chains can be incorporated in the arm in the same way. The cysteine comprises a free thiol group which can be used as reactive function for further grafting on solid support like amine group in the presence of SMCC. Similarly, other amino acid molecules can be incorporated in said side chain tail in order to obtain a longer arm, more hydrophobic or more hydrophilic or to introduce specific chemical groups.

The use of the carboxylic amino acid like the glutamic acid as the amino acid in the crossroad between the cyclic peptide and the anchorage tail is a key to the success for this invention. Unexpectedly, it can not be replaced by aspartic acid as it is the case for the formation of single cyclic peptide without tail. Indeed, in a first set of experiments, the Inventors have tried to obtain the synthesis of the tailed cyclic peptide *cyclo*[D-Val-Arg-Gly-Asp-Asp-(εAhx-Cys-NH₂)], with the protocol developed for the simple cyclic peptides (Kates *et al.*, 1993, *Anal. Biochem.*, 212, 303-310), incorporating Fmoc-L-Asp-OAl as cyclizing amino acid. The method failed to give the expected product as more than five different compounds were identified in the crude product. However, the same method provided excellent results for the preparation of the simple cyclic analogue *cyclo*[D-Val-Arg-Gly-Asp-Asn] which only differs by the absence of the anchoring side-chain tail -εAhx-Cys-

NH₂.

The analysis of the products obtained has shown that the aspartic acid incorporated in the sequence at the N-terminus of 6-aminohexanoic acid leads to the undesired side reaction of aspartimide formation in the presence of piperidine. This aspartimide formation on the aminoterminus of 6-aminohexanoic acid has not yet been described, especially with the allyl protecting group.

Furthermore, the formation of the aspartimide ring led to a product with the same molecular weight as the expected cyclic peptide, and prevented the reaction of the free terminal amino group for cyclization. This allowed additional side reactions to occur during the last step of the synthesis such as N-terminal acetylation or tetramethylguanidium formation by TBTU treatment.

These side reactions indicates that aspartic acid is unsuitable for the synthesis of tailed cyclic peptides. In a second set of experiments, the Inventors have replaced the aspartic acid by the glutamic acid. The synthesis of *cyclo*[D-Val-Arg-Gly-Asp-Glu-(εAhx-Cys-NH₂)] was performed according to the method of the invention, using Fmoc-L-Glu-OAl as cyclizing amino acid. Surprisingly, this method also failed to give satisfying

results, as the expected product ($M_r = 771.3$; $R_t = 19.6$ min) was largely contaminated by side reaction products. Most of these side products had a higher hydrophobicity and a higher molecular weight than 5 KD, except the product eluting at $R_t = 10.6$ min which was found to be 1-hydroxybenzotriazole (HOBt). The use of BOP instead of TBTU as cyclizing reagent gave essentially the same HPLC profile without HOBt. In another experiment, an additional manual washing step, including DMF, CHCl₃, methanol, CH₂Cl₂, 8% DIEA in DMF, 8% DIEA in CH₂Cl₂, was introduced in the synthesis protocol after palladium wash and Fmoc removal. This resulted in an improved crude product with the expected tailed cyclic peptide as major product of the reaction ($M_r = 771$; $R_t = 19.6$) and a minor side product corresponding to

the dimeric form of the peptide ($M_r = 1543.6$; $R_t = 30.2$ min). This indicated that the side products obtained in the first trials with Fmoc-L-Glu-OAl were the result of an incomplete washing of the resin during the last steps of the synthesis (allyl palladium cleavage and Fmoc removal). According to these experiments, the side reaction, identified as N-terminal acetylation, can be due to the presence of acetic acid in the solubilization solution of palladium(0) which is used for the allyl cleavage and which was probably not completely removed during the subsequent steps of the synthesis. Furthermore, after the final Fmoc removal (step (3) in Fig. 1), the carboxylate of the peptide is left as a piperidine salt which can be converted to piperidine amide under the coupling conditions of the cyclization step, adding +67 Da to the linear peptide. When the additional washing step was included in the protocol a non competitive cyclization was obtained.

The procedure was adapted for automatic synthe-

6

sis on the PerSeptive Biosystems 9050 Plus peptide synthesiser. Among the different washing conditions tested in the manual protocol, one selects the combined effect of solvent changing (dichloromethane in place of DMF) with the presence of an aminated base (DIEA 8%). The enclosed Table 1 details the automated washing conditions used after the allyl cleavage and the additional washing step after the palladium wash. These washing conditions were tested for the synthesis of *cyclo*-[DVal-Arg-Gly-Asp-Glu(ϵ Ahx-Cys-NH₂)] using TB-TU (2 x 3 h) or HATU (2 x 60 min) as cyclization reagents. The HPLC profile of the crude products presented in Figs 2A and 2B show only one main peak as for the manual washing. There is still very small amount of contaminating products which are lower when HATU was used for cyclization (Fig. 2B) compared with TB-TU (Fig. 2A). The yield obtained in these conditions was above 85% for the crude product and around 40% after purification.

The reason why such secondary products were obtained during the cyclization step of the tailed cyclic peptides, and not in the case of simple cyclic peptides, is still an open question, but this was systematically observed for different independent syntheses. Incomplete removal of acetic acid was also observed during the synthesis of the tailed cyclic peptide *cyclo*-[DVal-Arg-Gly-Asp-Asp(ϵ Ahx-Cys-NH₂)], giving rise to N-acetylated linear peptides with a molecular weight 42 kD higher than expected (Delforge D. et al., 1996. Lett. Pept. Sci., 3, 89-97). It is known that the formation of peptide secondary structures like *b*-sheets can be a major factor for the formation of impurities during solid-phase synthesis (Hyde C. et al., 1994. Int. J. Pept. Prot. Res., 43, 431-440). One can not exclude the formation of similar secondary structures during the synthesis of complex molecules like tailed cyclic peptides, and the possible interaction of these structures with some of the reagents which would then be difficult to remove with the standard washing steps of the procedure. If it is the case, this would explain the results obtained and the improvement achieved with the method of the invention.

The obtention of a cyclic peptide bearing a side chain tail can be obtained if the D-isomer of glutamic acid is used or another dicarboxylic amino acid with a longer side chain than glutamic acid, like the 2-amino adipic acid (D or L). Also the first or a subsequent amino acid which is incorporated during the synthesis of the peptide and which after release from the support will serve for further grafting can be any amino acid with function suitable for such use. This is the case for the amino group if lysine is used or the carboxyl group if aspartic or glutamic acid is used. These functional amino or carboxyl groups are easy to bind on activated support or proteins. These *tailed cyclic peptides* are ready-to-use for chemical grafting in various applications like the development of new biomaterials for improved cell adhesion, affinity chromatography, immunisation, vaccine development, ELISA kits or for building libraries of

conformationally constrained peptides.

A cyclic peptide bearing a RGD motif grafted on aminated poly(ethylene terephthalate) is given in the example 1 (Fig 3). In the example 2 (Figs 4, 5 and 6), said tailed cyclic peptide is grafted on bovine serumalbumin coated on polystyrene plates can serve as anchorage for the adhesion of cells. In these experiments, the tailed cyclic peptide was grafted on the BSA either before adsorption of the BSA on the plate (Figs 4A and 5) or after its adsorption on the plate (Figs 4B and 6). In one experiment, the tailed cyclic peptides were directly adsorbed on the BSA without any covalent coupling on the protein (Fig 7). These examples can be extended to any ligand which binds to a receptor. A peptide can be defined which mimics the ligand binding structure and which can take the place of the ligand on the receptor or enzyme binding site, and in this way obtain the desired biological response. This is especially useful for the preparation of biomaterial compatible with living cells. Such cyclic tailed peptides can also be constructed from non natural amino acids or using building blocks which can be incorporated in the amino acid sequence

The design of the peptide can also affect the cell behaviour, for example leading to a pro-adhesive surface as shown in example 2 for the tailed cyclic RGD peptide. However, other peptides could have different effects on cells. For example, REDV would be pro-adhesive, DGEA would be anti-adhesive, while SFLLR would enhance activation of cells, and ANFLV-WEIVRKPP would lower the activation of cells. Other peptides like TLEGTKKGHLHDYIGP would be proliferative, mainly due to the GHK aminoacids, while GHE and GHP would have anti-proliferative effects on endothelial cells.

One particular application of tailed cyclic peptides is to construct dimeric cyclic peptides using the tail for linking two peptides together. This is a particularly easy way to obtain dimeric cyclic peptides. The application of such construct are numerous. The most interesting is to obtain an agonist effect once the dimer is linked to the corresponding dimeric receptor. Indeed many receptors are active only in a dimeric form and this can be obtained with such dimeric cyclic peptide.

A peptidic tail can also be added to this cyclic dimeric peptide for its fixation on a support or a macromolecule such as a protein. Multimeric peptides can also be constructed in the same way by linking the tail of a cyclic peptide to the cyclic part of a second tailed cyclic peptide, leaving the tail of the second peptide free for another linking. An alternative is to provide on the peptide two tails ready for further linking.

The peptide sequence which is responsible for the binding of growth factors on their receptors is often known, and these peptides can advantageously be used as tailed cyclic peptides. For example, the CYAAPLK-PAKSC and CGTGYGSSSRRC are two peptides which can fix on the IGF-1 receptor. APSGHYKG and KRT-QYKL are specific for the bFGF receptor. ANFLV-

WEIVRKPP is specific for PDGF, and we propose a cyclic EQIMRIKPHQ for the receptor KDR of VEGF. These peptides can have antagonist effect when used as monomers, but agonist when dimeric as explained here above. An agonist effect of peptide mimicking the VEGF can be particularly useful for preparing biomaterial substitute for blood vessels since they could speed up the anchorage and the growth of endothelial cells, protecting this surface from platelet aggregation and other pro-inflammatory reactions. A proposed dimeric cyclic peptide with a tail ready for grafting on a polymeric surface is presented in the enclosed figure 7.

Example 1 : General description of the preparation process

Materials

Fmoc-protected amino acids and reagents, including Fmoc-L-Glu-OAI, Fmoc-PAL-EG-PS and solvents used for the synthesis were purchased from PerSeptive Biosystems (Watford Herts, UK). HATU used for on-line cyclization was a generous gift of PerSeptive Biosystems. Alternatively, some protected amino acids and TBTU coupling reagents were obtained from Neosystem Laboratoire (Strasbourg, France). All amino acids were used as free acids and activated just before coupling using TBTU. Except when otherwise mentioned in the text, tBu was used for side chain protection of Asp, while Trt and Pmc were used respectively as side chain protecting groups for Cys and Arg. All other reagents required for washings, cleavage and purification were obtained from Acros Chimica (Beerse, Belgium) except acetonitrile from Carlo Erba (Milano, Italy), N,N-dimethylformamide and diethylether from Labscan (Dublin, Ireland), tetrakis-(triphenylphosphine)-palladium(0) from Aldrich Chemical Company (Milwaukee, WI) and anisole from Sigma Chemical Company (St Louis, MO). All solvents were of HPLC grade or equivalent quality and were used without further purification except for TFA and dichloromethane which were *p.a.* grade and were redistilled before use.

Peptide synthesis by standard chain elongation

Automated continuous-flow solid-phase peptide synthesis was performed following a standard Fmoc protocol (Atherton E., and Sheppard R.C., 1989. Solid Phase Peptide Synthesis. A Practical Approach, IRL Press at Oxford University Press, Oxford) on a PerSeptive Biosystems 9050 Plus PepSynthesizer. The synthesiser was set in the "Allyl mode" which allows the synthesis of linear, branched or cyclic peptides (Kates S.A. et al., 1993. Anal. Biochem., 212, 303-310). All amino acids were incorporated as free acids using TBTU as coupling agent (Knorr R. et al., 1989, Tetrahedron Lett., 30, 1927-1930), without addition of HOBt which is not required for TBTU activation. The typical protocol used

for standard chain elongation was as follows: Fmoc deprotection with 20% piperidine in DMF (7 min), DMF washing (7 min), extended amino acid coupling (60 min), and DMF washing (4 min). The pump flow rate was 5 ml/min. Syntheses were carried out on a Fmoc-PAL-PEG-PS resin (0.4 to 1.2 g; substitution 0.16 mmol/g) (Albericio F. et al., 1990. J. Org. Chem., 55, 3730-3743) available from PerSeptive Biosystems, with a four-fold excess of Fmoc-amino acid and TBTU. TBTU was directly added as a solid in the amino acid vials and dissolved to a final concentration of 0.3 M with a solution of DIEA 8% in DMF just before coupling. Syringe 2 of the synthesiser was used for this purpose.

For the synthesis of TCP a protected Fmoc-cysteine was incorporated as the first amino acid of the sequence (Fig. 2) to allow subsequently end point grafting if required. Fmoc-6-aminohexanoic acid was then incorporated as the second amino acid of the sequence to introduce a 6-carbon spacer between the anchoring cysteine of the tail and the cyclizing amino acid residue of the peptidic cycle (Fig. 2). An α -allyl-protected Fmoc-glutamic acid residue Fmoc-L-Glu-OAI was coupled *via* its free carboxylic side-chain as third amino acid to allow cyclization to occur at the final step of the synthesis. The rest of the sequence elongation was achieved following standard Fmoc peptide synthesis, before allyl deprotection (**2**) and peptide cyclization (**4**) (Fig. 2).

Automated allyl deprotection and improvement of washing conditions after palladium treatment

Side chain removal of the allyl group was performed automatically on the column following the method previously described by Kates et al. for automated synthesis of cyclic peptides (Kates S.A. et al., 1993. Anal. Biochem., 212, 303-310). Pd(PPh₃)₄ used as catalyst (2 meq / meq of peptide) was stored in the amino acid rack after the last amino acid of the sequence. Syringe 3 of the synthesiser was used to dissolve the palladium catalyst to a final concentration of 0.07 M with a solution of 5% HOAc and 2.5% NMM in CHCl₃. After 2 h of recirculation through the column, this solution was washed with a solution of 0.5% DIEA and 0.5% diethyldithiocarbamate in DMF (auxiliary bottle, 12 min, 6 ml/min) and with DMF (10 min, 6 ml/min) to prepare the support for the final cyclization step. In the final synthesis experiments an additional wash cycle with DIEA 8% in CH₂Cl₂ (wash 2 bottle, 12 min, 6 ml/min) was introduced for complete removal of reagents and side products resulting from allyl deprotection and palladium wash (Table 1). The program of the software in "Allyl Mode" and "MCPS Mode" was adapted accordingly.

Automated cyclization

After the linear synthesis and allyl deprotection, the N-terminal Fmoc protecting group was removed by piperidine treatment (20% in DMF, 7 min) and the resin was

washed with DMF (7 min. 7 ml/min). The cyclization was achieved on the column by using a four-fold excess of coupling agent during different periods of time. Both TBTU (2×3 h) (Atherton E., and Sheppard R.C., 1989. Solid Phase Peptide Synthesis. A Practical Approach. IRL Press at Oxford University Press, Oxford), BOP (2×3 h) (Castro B. et al., 1975. Tetrahedron Lett., 1219-1222) and HATU (2×60 min) (Carpino L.A. et al., 1994. J. Chem. Soc. Chem. Commun., 201-203) have been tested as cyclization activators, without HOBr, as previously mentioned. Four equivalents of activator, stored in the amino acid vial positioned after the one containing the palladium catalyst, were dissolved to a final concentration of 0.3 M with a solution of DIEA 8% in DMF from syringe 2. The solution was delivered to the allyl-deprotected peptide support and was allowed to recirculate during a time long enough to obtain complete cyclization. This step was usually repeated a second time with fresh activator to be sure to achieve a complete cyclization. This was checked by the Kaiser ninhydrin test (Kaiser E.T. et al., 1970, Anal. Biochem., 34, 595-598) with an increased revelation time (15 min at 100°C) to compensate for the poor sensitivity of the test on PEG-PS beads. Finally, the resin was washed with DMF (4 min, 7 ml/min).

Cleavage conditions

The peptide-resin was removed from the column, washed with methanol, dichloromethane and dried under vacuum. Cleavage was achieved by treatment with reagent R (Albericio F. et al., 1990, J. Org. Chem., **55**, 3730-3743) *i.e.* TFA-thioanisole-ethanedithiol-anisole (90:5:3:2) for 2 h. The filtrate was collected and the resin was further washed with TFA. After evaporation of the solution under high vacuum, diethylether was added to the dry residue and cooled to -20°C to precipitate the peptide. The supernatant was removed and cold diethylether was further added to repeat the washing step up to five times, followed by extractions in biphasic water-ether (50:50) systems if necessary. The aqueous solution obtained was then lyophilised after addition of some drops of glacial acetic acid, to maintain the free thiols in their reduced state.

Characterisation of the synthesis product

Peptide purity was determined by reversed-phase HPLC on a Nucleosil C₁₈ column (250 mm x 4 mm) from Macherey-Nagel (Düren, Germany). The HPLC system was a Hewlett Packard HP 1090 Liquid Chromatograph equipped with a programmable wavelength diode array detector HP 1040 M Series II, set at 214 and 270 nm. Analyses were performed at a flow rate of 1 ml/min with a linear gradient of solvent A to solvent B, from 10 % B (at $t = 3$ min) to 50 % B (at $t = 60$ min), where A was TFA 0.1% in H₂O and B was TFA 0.1% in CH₃CN-H₂O (80:20). Samples were solubilized in solvent A at a concen-

tration of 1 mg/ml. Injection volume was 50 ml. Molecular weights were determined by high performance liquid chromatography / mass spectrometry (HPLC-MS) (Heath T.C. and Giordani A.B., 1992). Chromatogr.

- 5 638. 9-19; Wang R., and Chait B.T., 1994, Curr. Opin. Biotechnol., 5, 77-84) on a Kontron Instruments HPLC 325 System connected to a uv-detector Kontron 332 and a Fisons Instruments VG platform II electrospray ionisation mass spectrometer (ESI-MS).

Example 2 : Grafting of tailed cyclic peptides on aminated poly(ethyleneterephthalate)

Poly(ethyleneterephthalate) (PET) film samples (di-

- 15 ameter 1.2 cm) were first carboxylated by hydrolysis in
0.25 N NaOH and acetonitrile (1:1: V/V) and subsequent
oxidation with permanganate according to Marchand-
Brynaert *et al.* (1995. J. Colloid Interface Sci., **173**,
20 236-244). Carboxylated PET was then aminated ac-
cording to the method of Zammateo *et al.* (1996. Anal.
Biochem., **236**, 85-94) using ethylenediamine instead of
N-methyl-1,3-propane diamine to introduce primary
amino groups on the support. The tailed cyclic peptide
25 *cyclo[*-DVal-Arg-Gly-Asp-Glu(-εAhx-Tyr-Cys-NH₂)-]*]*
was iodinated with ¹²⁵I according to Greenwood *et al.*
(1963. Biochem J., **89**, 114-123) and grafted on the am-
20 imated PET using *N*-succinimidyl 4-(*N*-maleimidome-
thyl)cyclohexane-1-carboxylate (SMCC) as bifunctional
reagent. Samples of aminated PET were incubated 30
30 min at 30°C under shaking in 3.7 mM SMCC 100 mM
NaH₂PO₄ pH 7.0 10 % DMSO (2 ml/sample). Activated
support was washed 3 x 5 min with NaH₂PO₄ pH 6.0
and incubated 20 hours at room temperature under
35 shaking in the presence of a 10⁻⁶ M solution of ¹²⁵I-la-
belled tailed cyclic peptide in 100 mM NaH₂PO₄ pH 6.0
(2 ml/sample). Samples were washed 3 x 5 min with 100
mM NaH₂PO₄ pH 6.0 and 3 x 5 min with H₂O. The ra-
dioactivity associated to each individual sample was
40 counted by liquid scintillation counting and was reported
to the surface of the sample to determine the amount of
iodinated tailed cyclic peptide grafted by surface unity
(picomol of peptide grafted/cm²) (Fig 3). The results ob-
tained show an increased grafting of the peptide when
45 a covalent attachment is performed using SMCC on am-
minated PET films compared to simple adsorption on am-
minated PET or coupling on native (unmodified) PET
films.

Example 3 : Enhancement of cell adhesion on polystyrene microwells coated with bovine serumalbumin (BSA) bearing tailed cyclic peptides containing the Arg-Gly-Asp (RGD) sequence

In the first experiment, the wells of a 96-wells poly-

- 55 styrene microwells plate were coated with bovine serum albumin already grafted with a RGD tailed cyclic peptide before testing the adherence of these coated plates (Fig. 4A). Albumin was activated with the heterobifunc-

tional reagent sulfo-succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate *i.e.* sulfo-SMCC, to introduce reactive maleimido groups at the surface of the protein. BSA (8 mg/ml) was incubated in 100 mM NaH₂PO₄ pH 7.2 during 1 h at room temperature in the presence of sulfo-SMCC at a final concentration of 1.8 mg/ml. The solution was desalting and reconcentrated at 1 mg/ml on Amicon PM10. The activated BSA was then incubated overnight at room temperature in the presence of *cyclo*[D-Val-Arg-Gly-Asp-Glu(-εAhx-Tyr-Cys-NH₂)] at a concentration of 1.6 mg/ml. The BSA-peptide conjugate was desalting and reconcentrated at 1 mg/ml. The wells of a polystyrene microwell plate were then coated with this conjugate by incubation of 100 ml/well during 60 min, before washing two times with PBS.

In the second experiment the wells were coated with native bovine serum albumin which was then grafted with the tailed cyclic RGD peptide (Fig. 4B). The wells of a 96-well polystyrene microwell plate were first coated with bovine serum albumin (BSA) by incubating the protein at a concentration of 1 mg/ml (100 ml/well) during 60 min in PBS buffer. The wells were then washed two times with PBS buffer before activation with succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC). SMCC was dissolved at a final concentration of 26 mg/ml in 100 mM NaH₂PO₄ pH 7.2 10 % DMSO and incubated in BSA-activated microwells (100 ml/well) at room temperature during 60 min. The wells were then washed two times with 100 mM NaH₂PO₄ pH 7.2 to remove unreacted SMCC. The tailed cyclic peptide *cyclo*[D-Val-Arg-Gly-Asp-Glu(-εAhx-Tyr-Cys-NH₂)] at a concentration of 0.18 mg/ml in 100 mM NaH₂PO₄ pH 7.2 (100 ml/well) was incubated in the microwells overnight at room temperature. The wells were washed two times with phosphate buffer to remove the excess of peptide (unreacted). In a last experiment BSA was adsorbed on the plates as above and then incubated in the presence of the peptides for 15 h before washing and to performed the adhesion experiment with the cells.

Cell adhesion assays were performed on 96-well polystyrene plates coated with BSA bearing pro-adhesive Arg-Gly-Asp (RGD)-containing tailed cyclic peptides. Endothelial cells in MCDB were incubated 15 min at 37°C, then washed two times with PBS buffer. Adhesion of the cells in the wells was estimated by the colorimetric method of Givens K.T. *et al.* (1990, Invest. Ophthalmol. Visual Sci., 31, 1856-1862) by measuring the absorbance at 405 nm of the para-mitrophenol liberated after incubating para-nitrophenol-N-acetyl-B-D-glucosaminidase. In both experiments, the results presented in figures 5 and 6 showed an increased cell adhesion on polystyrene coated with BSA bearing the RGD tailed cyclic peptide compared to unmodified polystyrene or polystyrene coated with BSA. Control experiments were performed with BSA bearing cysteine residues grafted via SMCC (negative control) or with linear peptide, or with a random RGD peptide, or with fibronectin adsorbed on polystyrene support (positive control).

The results showed better cell adhesion obtained for the adsorption of BSA bearing RGD tailed cyclic peptides than for the adsorption of the proadhesive protein fibronectin.

5

Claims

1. Cyclic peptide comprising a dicarboxylic amino acid and bearing at least one tail for subsequent coupling on a element selected from the group consisting of a solid support, a high molecular weight compound, a marker, one or more other similar or different cyclic peptides or a mixture thereof.
2. A cyclic peptide according to claim 1, characterised in that the dicarboxylic amino acid is a D or L amino acid, selected from the group consisting of the glutamic acid and the 2-amino adipic acid.
3. A cyclic peptide according to the claim 1 or 2, wherein the tail is made of amino acids units, possibly separated by one or more spacers.
4. A cyclic peptide according to claim 3, characterised in that the amino acids units of the tail are selected from the group consisting of cysteine, lysine, aspartic acid, glutamic acid or a mixture thereof.
5. A cyclic peptide according to claim 3 or 4, characterised in that the spacer is a 6-amino-hexanoil.
6. A cyclic peptide according to any of the preceding claims, comprising in its cycle a sequence which mimics one binding sequence of a growth factor, preferably the vascular endothelial growth factor (VEGF).
7. A cyclic peptide according to claim 6, characterised in that said sequence is the sequence SEQ ID NO. 1 : -EQIMRIKPHQ.
8. Preparation process of the cyclic peptide according to any of the preceding claims, by a chemical synthesis using an automatic peptide synthesiser.
9. Process according to claim 8, characterised in that the cyclic peptide is obtained by chemical synthesis on a solid support or a high molecular weight compound.
10. Process according to claim 9, characterised in that the step comprises the use of dichloromethane as a solvent in the presence of an aminated base in the washing step after the step of allyl cleavage and palladium wash.
11. Biomaterial comprising the cyclic peptide according

to any of the claims 1 to 7.

5

10

15

20

25

30

35

40

45

50

55

10

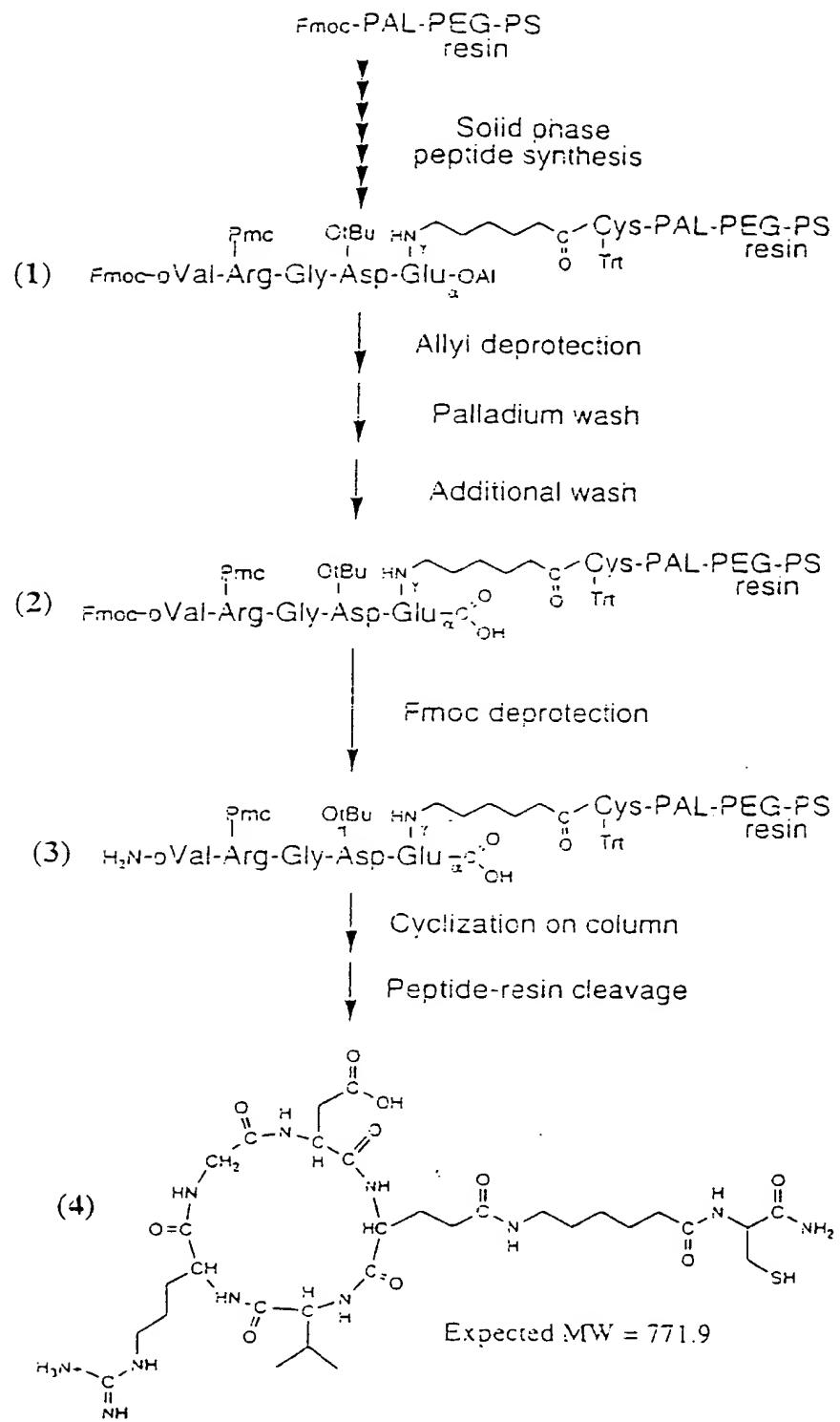


FIG. 1

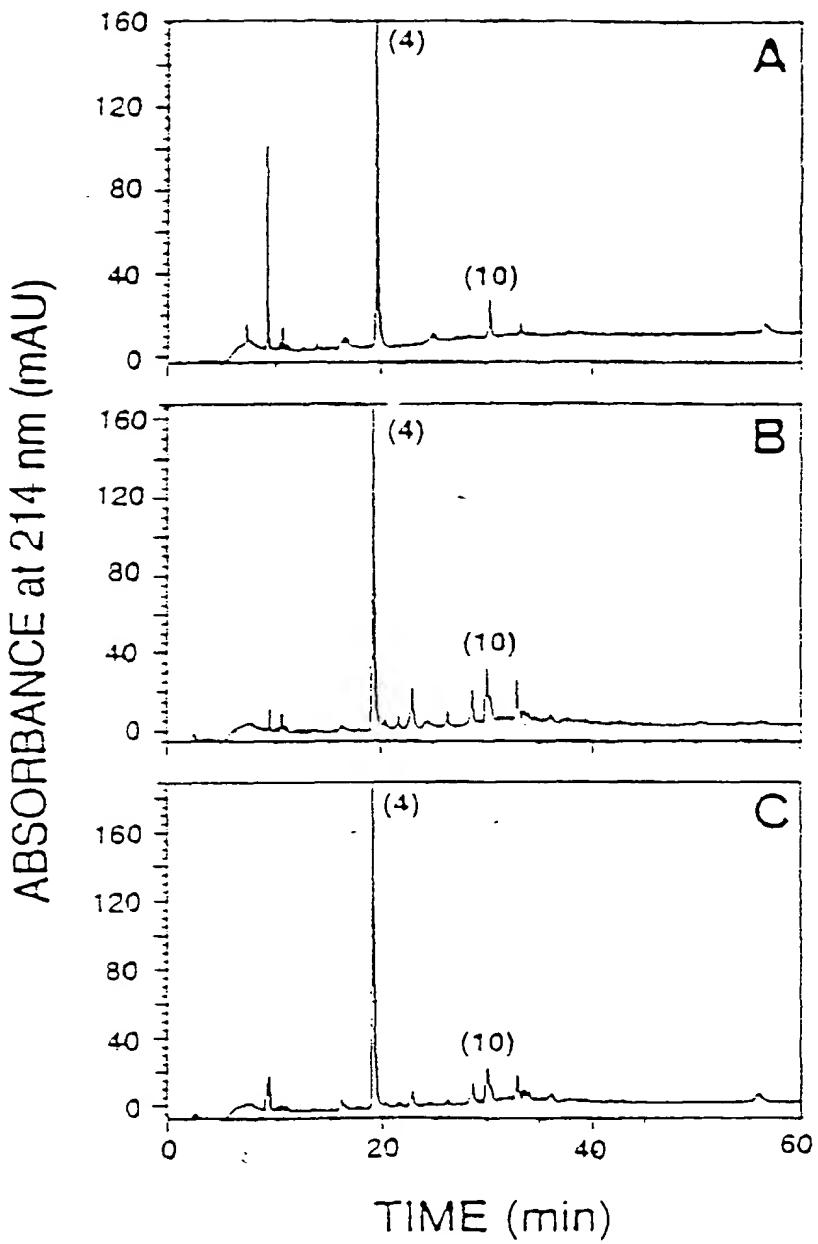


FIG. 2

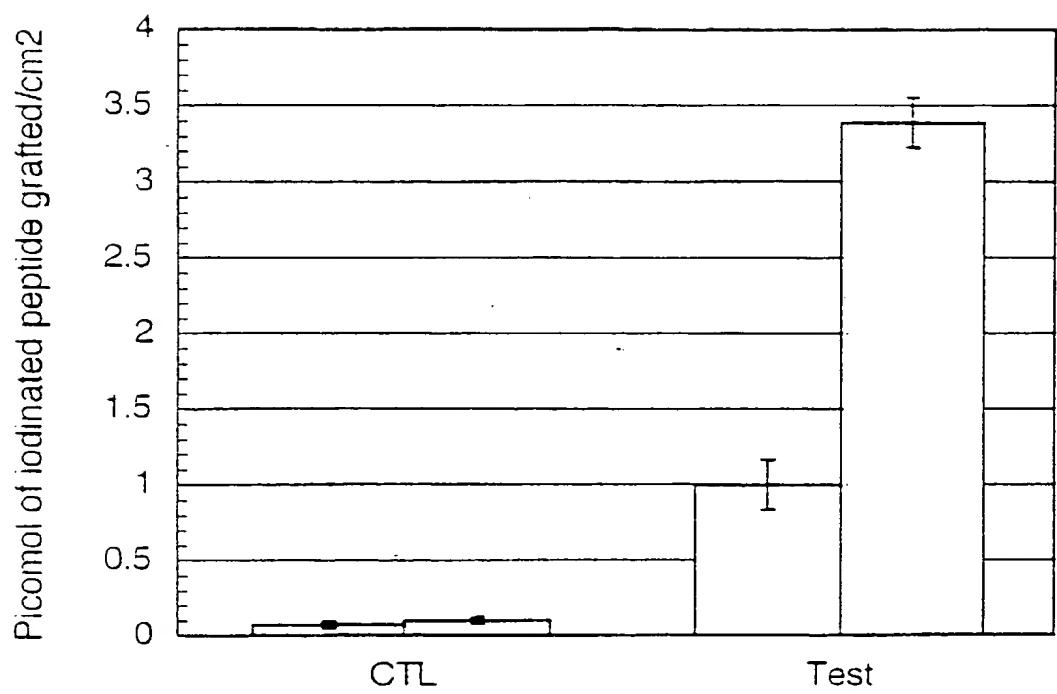


FIG. 3

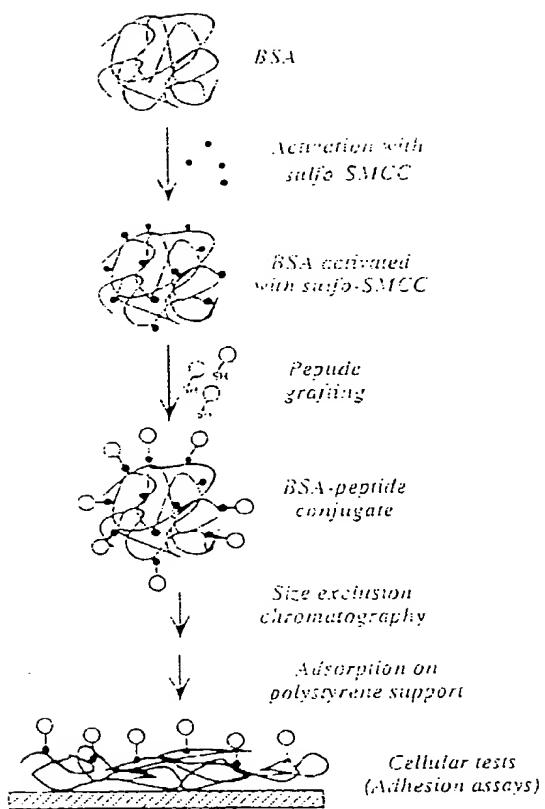


FIG. 4A

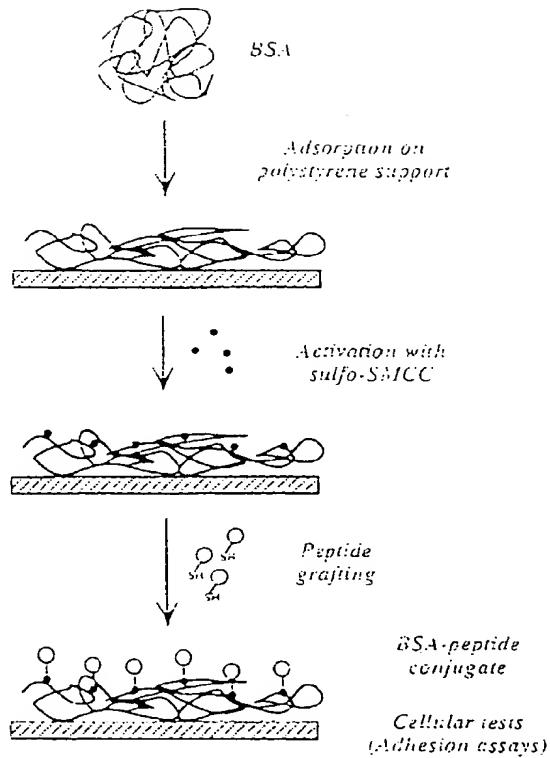


FIG. 4B

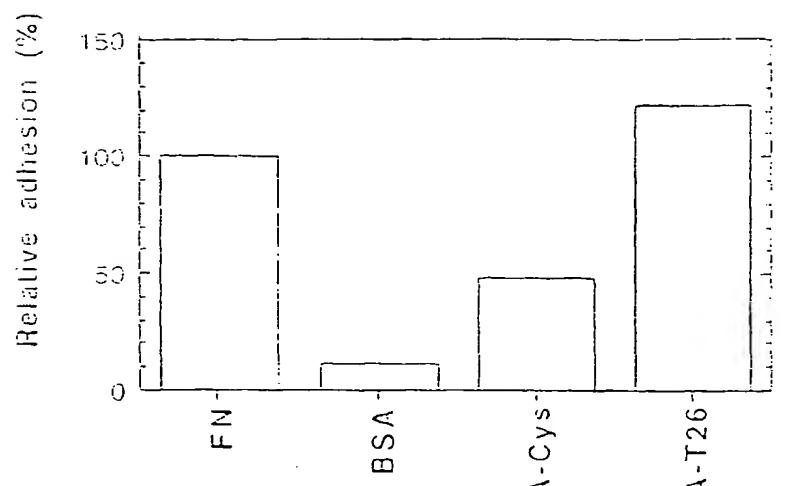


FIG. 5

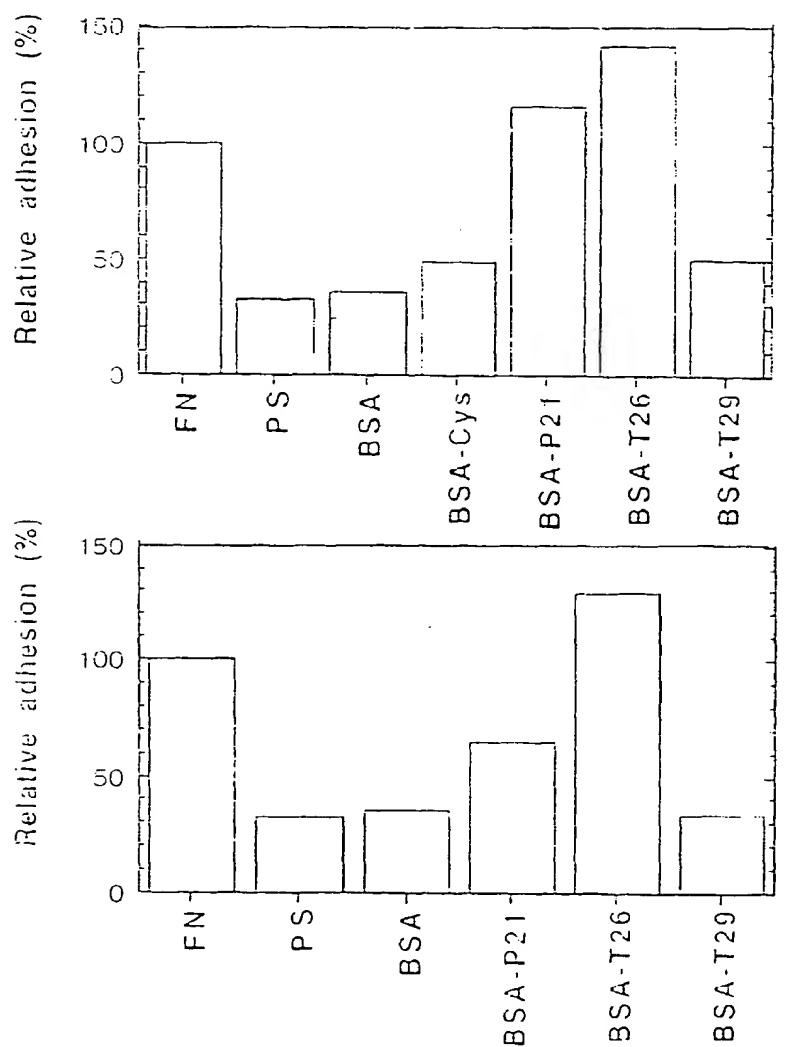


FIG. 6

FIG. 7

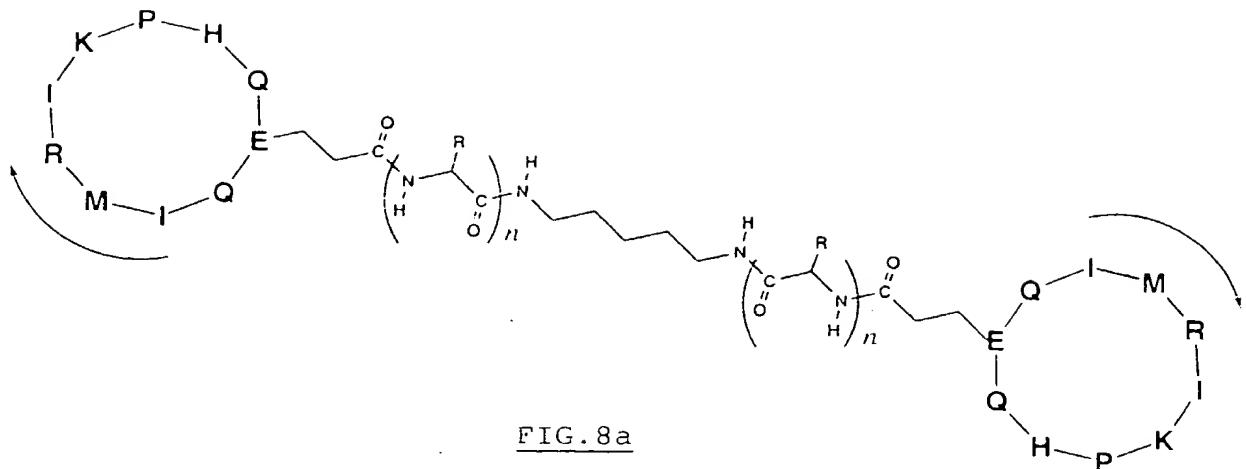


FIG. 8a

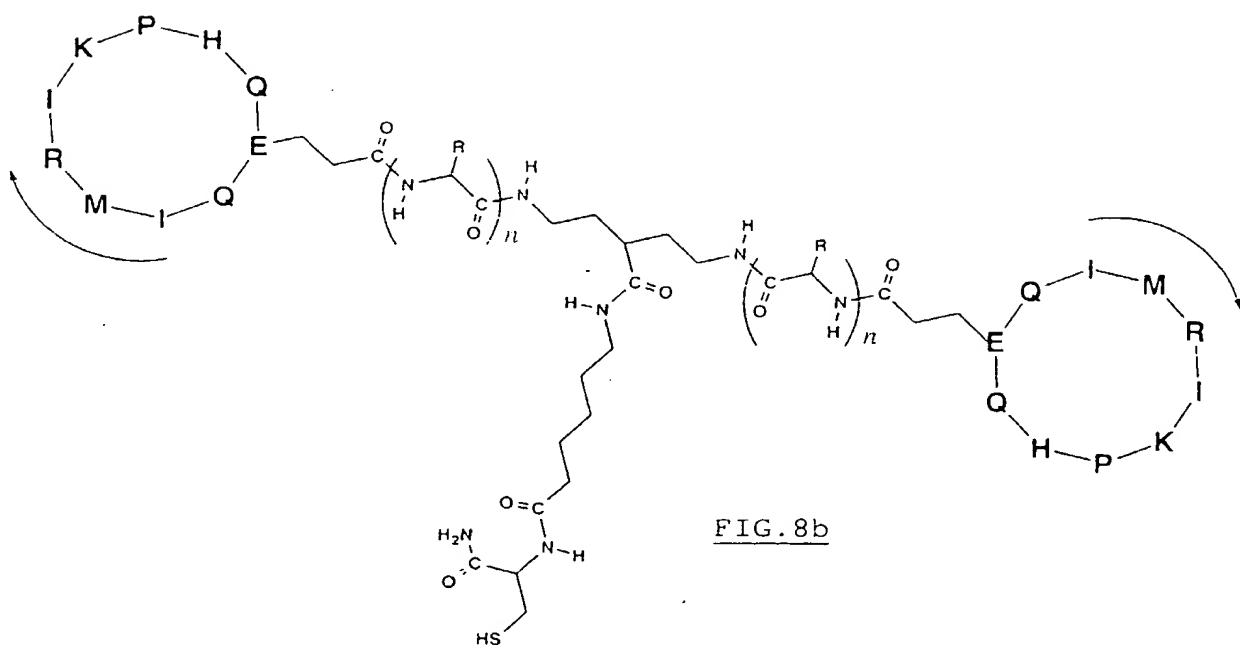


FIG. 8b

(19)



Europäisches Patentamt
European Patent Office
Office européen des brevets

(11)



EP 0 844 252 A3

(12)

EUROPEAN PATENT APPLICATION

(88) Date of publication A3:
08.07.1998 Bulletin 1998/28

(51) Int Cl.⁶: **C07K 7/64, C07K 17/00**

(43) Date of publication A2:
27.05.1998 Bulletin 1998/22

(21) Application number: **97870182.9**

(22) Date of filing: **14.11.1997**

(84) Designated Contracting States:
**AT BE CH DE DK ES FI FR GB GR IE IT LI LU MC
NL PT SE**

Designated Extension States:
AL LT LV MK RO SI

(30) Priority: **15.11.1996 US 30980 P**

(71) Applicant: **Remacle, José
5020 Malonne (BE)**

(72) Inventors:

- **Remacle, José
5020 Malonne (BE)**
- **Delforge, Dominique
5000 Namur (BE)**

(74) Representative: **Van Malderen, Joelle et al
Office Van Malderen,
Place Reine Fabiola 6/1
1083 Bruxelles (BE)**

(54) **Cyclic peptides bearing a tail designed for subsequent chemical coupling and process for preparing same**

(57) The present invention relates to a cyclic peptide comprising a dicarboxylic amino acid, said cyclic peptide bearing at least one tail for subsequent coupling on a solid support, on a high molecular weight compound,

on a marker, or on one or more other similar or different cyclic peptides.

The present invention is also related to the preparation process of said cyclic peptide and to a biomaterial comprising said cyclic peptide.

EP 0 844 252 A3



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 97 87 0182

DOCUMENTS CONSIDERED TO BE RELEVANT									
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)						
X	D. DELFORGE ET AL: "Solid phase synthesis of tailed cyclic peptides." LETTERS IN PEPTIDE SCIENCE, vol. 3, no. 2, May 1996, pages 89-97, XP002063392 * the whole document * * page 96, last paragraph * * page 90, column 1, paragraph 2 * ---	1-5,8-11	C07K7/64 C07K17/00						
X	M. ART ET AL.: "Synthesis of linear and cyclic RGD-peptides for the purpose of their chemical grafting on poorly adhesive polymers such as polyethylene terephthalate" POLYM. PREPRINTS, vol. 36, no. 1, April 1995, pages 115-116, XP002063393 * the whole document * * page 115, column 2, last paragraph - page 116, column 1, paragraph 1 * ---	1-3,5, 8-11							
P,X	D. DELFORGE ET AL.: "Automated Solid-Phase synthesis of cyclic peptides bearing a Side-chain tail designed for subsequent chemical grafting" ANALYTICAL BIOCHEMISTRY, vol. 242, 15 November 1996, pages 180-186, XP002063394 * the whole document * ---	1-5,8-11	TECHNICAL FIELDS SEARCHED (Int.Cl.6) C07K						
<p>The present search report has been drawn up for all claims</p> <table border="1"> <tr> <td>Place of search</td> <td>Date of completion of the search</td> <td>Examiner</td> </tr> <tr> <td>THE HAGUE</td> <td>7 May 1998</td> <td>Cervigni, S</td> </tr> </table>				Place of search	Date of completion of the search	Examiner	THE HAGUE	7 May 1998	Cervigni, S
Place of search	Date of completion of the search	Examiner							
THE HAGUE	7 May 1998	Cervigni, S							
CATEGORY OF CITED DOCUMENTS		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons S : member of the same patent family, corresponding document							
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document									



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number

EP 97 87 0182

DOCUMENTS CONSIDERED TO BE RELEVANT									
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)						
X	S.A. KATES: "Automated Allyl cleavage for continuous-flow synthesis of cyclic and branched peptides" ANALYTICAL BIOCHEMISTRY, vol. 212, 1993, pages 303-310, XP002063395 * the whole document * * page 305, column 2, last paragraph - page 306, column 1, paragraph 1 * ---	1,8-11							
X	B. IVANOV: "Synthesis and use of a new Bromoacetyl-derivatised heterotrifunctional amino acid for conjugation of cyclic RGD-containing peptides" BIOCONJUGATE CHEM., vol. 6, 1995, pages 269-277, XP002063396 * the whole document * * scheme 1,2 and 3 * ---	1,8,9,11							
X	H.F. BRUGGHE ET AL.: "Simultaneous multiple synthesis and selective conjugation of cyclised peptides derived from a surface" INTERNATIONAL JOURNAL OF PEPTIDE AND PROTEIN RESEARCH, vol. 43, 1994, COPENHAGEN DK, pages 166-172, XP002063397 * the whole document * * figure 2 * ---	1,8,9,11	TECHNICAL FIELDS SEARCHED (Int.Cl.6)						
A	DD 150 199 A (JAKUBKE HANS DIETER; NEUBERT KLAUS; OEHME PETER; BERGMANN JUTTA; HARTRO) * the whole document * ---	-/-							
<p>The present search report has been drawn up for all claims</p> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 33%;">Place of search</td> <td style="width: 33%;">Date of completion of the search</td> <td style="width: 34%;">Examiner</td> </tr> <tr> <td>THE HAGUE</td> <td>7 May 1998</td> <td>Cervigni, S</td> </tr> </table> <p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>				Place of search	Date of completion of the search	Examiner	THE HAGUE	7 May 1998	Cervigni, S
Place of search	Date of completion of the search	Examiner							
THE HAGUE	7 May 1998	Cervigni, S							



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number

EP 97 87 0182

DOCUMENTS CONSIDERED TO BE RELEVANT									
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)						
A	<p>WO 95 21193 A (UNIV MCGILL ; SARAGOVICH H (CA); LESAUTEUR LYNNE (CA); CUELLO A C) * the whole document *</p> <p>-----</p>								
TECHNICAL FIELDS SEARCHED (Int.Cl.6)									
<p>The present search report has been drawn up for all claims</p> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 33%;">Place of search</td> <td style="width: 33%;">Date of completion of the search</td> <td style="width: 34%;">Examiner</td> </tr> <tr> <td>THE HAGUE</td> <td>7 May 1998</td> <td>Cervigni, S</td> </tr> </table>				Place of search	Date of completion of the search	Examiner	THE HAGUE	7 May 1998	Cervigni, S
Place of search	Date of completion of the search	Examiner							
THE HAGUE	7 May 1998	Cervigni, S							
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>									